REMARKS

Claims 7-26 were pending in the application. Claims 23 and 26 have been cancelled as they are drawn to a non-elected invention. Claims 8, 10, 11,12, 20 21, and 24 have been amended. A **"Version With Markings to Show Changes Made"** is attached hereto as Appendix A. Accordingly, claims 7-22, 24 and 25 will be pending upon entry of the amendment. For the Examiner's convenience, all of the pending claims are listed in Appendix B.

In compliance with 37 C.F.R. §1.72(b), an Abstract for the above-identified application is being submitted concurrently on a separate page.

Support for the amendments can be found throughout the specification and claims as originally filed. For example, support for the amendment to claim 8 can be found in the specification at least at page 68, line 13; support for the amendment of claim 10 can be found in the specification at least at page 65, line 26 through page 66, line 3, and page 67, lines 4-5 and Table 9 condition I; support for the amendment of claim 11 can be found in the specification at least at page 65, lines 9-15; support for the amendment of claim 12 can be found in the specification at least at page 66, lines 6-12 and on page 67 conditions A-F shown in Table 9 and ; support for the amendment of claim 20 can be found in the specification at least at page 65, lines 1-15; support for the amendment of claim 21 can be found in the specification at least at page 3, line 20, page 10, Table 1, and to page 46, line 20 through page 48, line 3; and support for the amendment of claim 24 can be found in the specification at least at page 62, lines 3-7.

No new matter has been added by the amendments. Any amendments to and/or cancellation of the claims should in no way be construed as acquiescence to any of the Examiner's rejections and was done solely to expedite prosecution. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Claim Rejections – 35 U.S.C. §101

established utility. Specifically the Office Action states that "the instant specification

does not teach any significance or functional characteristics of the human polynucleotide (SEQ ID NO: 11) or polypeptide (SEQ ID NO: 1) (Paper No. 14, page 4). Applicants respectfully traverse this rejection.

Applicants respectfully submit that a credible, specific and substantial utility is immediately apparent from Applicants' specification and the knowledge in the art at the time of Applicants' invention. Specifically, Applicants teach that the claimed nucleic acids, which were isolated from a cDNA library from human stomach cancer tissue, encode a polypeptide that is a transmembrane protein. At the time the instant applications was filed, it was well recognized by those skilled in the art that membrane proteins play important roles, for example, as signal receptors, ion channels, and transporters in the material transportation and the information transmission which are mediated by the cell membrane. The importance of membrane proteins in physiological homeostasis has led numerous groups to the search for new genes encoding further members of this functionally important class of molecules. Accordingly, as described in the specification, for example, at pages 8-9 and 42-43 Applicants developed a strategy to selectively identify and characterize those polynucleotides which encode for transmembrane proteins. This strategy was successful, and the resulting polynucleotide sequences, disclosed in Applicants' application, encode proteins that comprise certain structural and functional features, e.g., hydrophobic and hydrophilic profiles, which are intrinsic to the class of genes which encode proteins encoding a transmembrane domain. Thus, specific structural or functional information for the proteins corresponding to the polynucleotide sequences of the invention was disclosed *a priori*.

With regard to the presently claimed nucleic acids and polypeptides (SEQ ID No: 1 and SEQ ID N0: 11), Applicants further disclosed that the protein is analogous to the chicken stem cell antigen 2 (Sca-2; GenBank Accession No. L34554; Appendix C). Sca-2 is a member of the Ly-6 family of cell surface proteins, a group of molecules differentially expressed in several hematopoietic lineages that have been shown to function in signal transduction and cell activation (see, e.g., Classon *et al.* PNAS

family of membrane proteins.

Indeed, Reiter *et al.* (PNAS 95:1735-1740, 1998, Appendix E) describe a gene that is overexpressed in prostate cancer tissue which they named prostate stem cell antigen (PSCA) based on its strong homology to Sca-2 (see p 1736, column 2, second paragraph). As shown in Appendix F, the alignment of PSCA with the amino acid sequence set forth in SEQ ID No:1 of the instant application indicates that these two proteins are 99.2% identical. Specifically, these two polypeptides differ by a single amino acid and, for example, could be allelic variants of the same gene. Accordingly, Applicants respectfully submit Appendices E and F as an example of post-filing evidence which supports the credibility of Applicants' assertions of the biological activity and/or utility of the claimed nucleic acid molecules and polypeptides.

In view of the foregoing, Applicants respectfully request that the Examiner reconsider and withdraw this section 101 rejection.

Claim Rejections - 35 U.S.C. § 112, First Paragraph

Claims 7-22 and 24-25 are rejected on the ground that one skilled in the art would not know how to use the claimed invention because the claimed invention is not supported by either a specific and substantial utility or a well established utility.

Applicants respectfully traverse this rejection. As discussed above, the claim subject matter has a well-established utility and, thus, one of ordinary skill in the art would know how to use the claimed invention given the guidance provided in the instant specification. Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

Claims 8, 10-20 and 24-25 were rejected on the ground that the specification "does not teach functional or structural characteristics of the polynucleotides and polypeptides in the context of a cell or organism. The description of one polynucleotide species (SEQ ID NO: 11) and one polypeptide species (SEQ ID NO: 1) is not adequate written description of an entire genus of functionally equivalent polynucleotides and polypeptides which incorporate all variants and fragments"(Paper No. 14, page 13).

known characteristics. The specification provides ample guidance for making and using

nucleic acids and polypeptides within the scope of the pending claims. For example, see pages 38-46 of the specification, and pages 61-68 line 17 of the specification. Accordingly, Applicants respectfully request reconsideration and withdrawal of this rejection.

Claim Rejections - 35 U.S.C. § 112, Second Paragraph

Claims 12, 20-22 and 24-25 were rejected as indefinite on the ground that "[I]n the absence of a recitation of clear hybridization conditions (e.g., "hybridizes at wash conditions of A X SSC and B % SDS at C°"), the claims fail to define the metes and bounds of the varying structures of polynucleotides recited in the claimed methods.

Applicants respectfully submit that the rejection of claims 12, 20-22, and 24-25 has been obviated by the amendment of these claims to include the stringency conditions, as suggested by the Examiner. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

Claims 20-22 and 24-25 were also rejected as indefinite "because a claim that depends from a claim which 'consists of' the recited elements or steps cannot add an element or step."

Applicants have amended claim 20 so that it is no longer in Markush format, and have put claim 21 into independent form. Accordingly, this rejection has been obviated reconsideration and withdrawal thereof is respectfully requested.

Claims rejections - 35 USC §102

Claims 8 and 12-19 were rejected as being anticipated by Marra et al. (Accession Number W42223, direct submission, EST database, 1 September 1996).

Marra *et al.*, teach an EST that is 294 nucleotides long which contains a sequence which is identical to 25 nucleotides of SEQ ID NO.: 11. Applicants respectfully submit that the rejection of claim 8 and hence the dependent claims12-19, which depend on claim 8 has been rendered moot in view of the amendment of this claim to specify that

rejection be reconsidered and withdrawn.

Claims 20-22 and 24-25 are rejected as being anticipated by Friedman et al. (Accession Number AAA41546, SPTREMBL database, 27 April 1993).

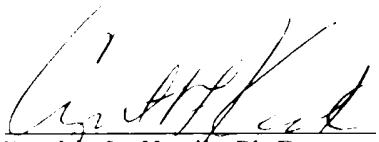
Accession No. AAA41546 teaches a polypeptide sequence of 135 amino acids which comprises ***6 amino acids*** of SEQ ID NO:1. As now amended, claim 20 is drawn to an isolated polypeptide encoding a fragment of ***at least 8 amino acid residues*** of the amino acid sequence of SEQ ID NO:1. Accordingly, the claimed subject matter is not anticipated by Friedman *et al.*, and Applicants respectfully request that this rejection be reconsidered and withdrawn.

CONCLUSION

Reconsideration and allowance of all the pending claims is respectfully requested. If a telephone conversation with Applicants' attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP



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Boston, MA 02109
Tel. (617) 227-7400
Fax (617) 742-4214
Dated: **December 13, 2001**

Appendix A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Lines 1-4 of the specification have been amended as follows:

~~DESCRIPTION~~

A HUMAN~~S~~ PROTEIN~~S HAVING~~ WITH TRANSMEMBRANE DOMAINS AND DNA~~S~~ ENCODING THES~~E~~ PROTEIN~~S~~

The paragraph starting at page 17, line 26 has been amended as follows:

Using the proteins of the invention it may also be possible to regulate immune responses in a number of ways. Down regulation may be in the form of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response. The functions of activated T cells may be inhibited by suppressing T cells responses or by inducing specific tolerance in T cells, or both. Immunosuppression of T cell responses is generally an active, non-antigen-specific, process which requires continuous exposure of the T cells to the suppressive agent. Tolerance, which involves inducing non-responsiveness or anergy in T cells, is distinguishable from immunosuppression in that it is generally antigen-specific and persists after exposure to the tolerizing agent has ceased. Operationally, tolerance can be demonstrated by the lack of a T cell response upon ~~reexposure~~ re-exposure to specific antigen in the absence of the tolerizing agent.

The paragraph starting at page 38, line 27 has been amended as follows:

~~The~~ Cells from the histiocyte lymphoma cell line U937 (ATCC CRL 1593) stimulated with phorbol ester, ~~tissues of~~ cells from human stomach cancer tissues delivered by operation, and ~~the~~ cells from human liver were used ~~for human cells~~ to extract mRNAs. The cell line was incubated by a conventional procedure.

In the Claims:

8. **(Amended)** An isolated nucleic acid ~~molecule~~ comprising ~~a fragment of the~~ a nucleotide sequence that is at least 95% identical to the nucleotide sequence set forth in SEQ ID NO:11~~, wherein the fragment comprises at least 10 nucleotides~~.

10. **(Amended)** An isolated nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide ~~comprising~~ consisting of the amino acid sequence set forth in SEQ ID NO:1~~.~~ , wherein the nucleic acid hybridizes in 4X SSC at 67° C, followed by one or more washes in 1X SSC, at 67° C to a complement of a nucleic acid having a nucleotide sequence set forth in SEQ ID NO:11.

11. **(Amended)** An isolated nucleic acid molecule which encodes a fragment of a polypeptide ~~comprising the~~ consisting of the amino acid sequence of SEQ ID NO:1, wherein the fragment comprises at least ~~5~~ 8 contiguous amino acid residues set forth ~~of~~ in the amino acid sequence of SEQ ID NO:1.

12. (New) An isolated nucleic acid molecule which hybridizes to the nucleic acid molecule of any one of claims 7, 8, 9, 10, or 11 ~~under stringent conditions.~~ in 1X SSC at 65° C, followed by one or more washes in 0.3X SSC at 65° C.

20. **(Amended)** ~~An isolated polypeptide selected from the group consisting of:~~

~~a) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:1, wherein the fragment comprises at least 5 contiguous amino acids of SEQ ID NO:1; and~~

~~b) a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:1, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:11 under stringent conditions.~~ A fragment of a polypeptide consisting of the amino acid sequence set forth in SEQ ID NO:1, wherein said fragment comprises 8 or more

21. **(Amended)** ~~The~~ An isolated polypeptide comprising of ~~claim 20 comprising~~ the amino acid sequence set forth in ~~of~~ SEQ ID NO:1.

~~23. An antibody which selectively binds to a polypeptide of claim 20.~~

24. **(Amended)** A composition comprising the polypeptide of any one of claims ~~7 to 11 and~~ 20, 21, and 22 and a pharmaceutically acceptable carrier.

~~26. A method for detecting the presence of a polypeptide of claim 18 in a sample comprising:~~

~~a) contacting the sample with a compound which selectively binds to the polypeptide; and~~

~~b) determining whether the compound binds to the polypeptide in the sample to thereby detect the presence of a polypeptide of claim 18 in the sample.~~

Appendix B

CLAIMS PENDING

7. An isolated nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID NO:11.

8. An isolated nucleic acid comprising a nucleotide sequence that is at least 95% identical to the nucleotide sequence set forth in SEQ ID NO:11.

9. An isolated nucleic acid molecule encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:1.

10. An isolated nucleic acid which encodes a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence set forth in SEQ ID NO:1, wherein the nucleic acid hybridizes in 4X SSC at 67° C, followed by one or more washes in 1X SSC, at 67° C to a complement of a nucleic acid having a nucleotide sequence set forth in SEQ ID NO:11.

11. An isolated nucleic acid molecule which encodes a fragment of a polypeptide consisting of the amino acid sequence of SEQ ID NO:1, wherein the fragment comprises at least 8 contiguous amino acid residues set forth in the amino acid sequence of SEQ ID NO:1.

12. An isolated nucleic acid molecule which hybridizes to the nucleic acid molecule of any one of claims 7, 8, 9, 10, or 11 in 1X SSC at 65° C, followed by one or more washes in 0.3X SSC at 65° C.

13. An isolated nucleic acid molecule consisting a nucleotide sequence which is complementary to the nucleotide sequence of the nucleic acid molecule of any one of

14. An isolated nucleic acid molecule comprising the nucleic acid molecule of any one of claims 7, 8, 9, 10, or 11, and a nucleotide sequence encoding a heterologous polypeptide.

15. An isolated nucleic acid molecule of claims 7, 8, 9, 10, or 11, wherein said nucleic acid molecule is operably linked to at least one expression control sequence.

16. A process for producing a protein encoded by the nucleic acid molecule of claim 15, which comprises:

(a) growing a culture of a host cell transformed with a vector comprising the polynucleotide of claim 15 in a suitable culture medium; and

(b) purifying said protein from the culture.

17. A vector comprising the nucleic acid molecule of any one of claims 7, 8, 9, 10, or 11.

18. The vector of claim 17, which is an expression vector.

19. A host cell transfected with the expression vector of claim 17.

20. A fragment of a polypeptide consisting of the amino acid sequence set forth in SEQ ID NO:1, wherein said fragment comprises 8 or more contiguous amino acid residues of the amino acid sequence set forth in SEQ ID NO:1.

21. An isolated polypeptide comprising of the amino acid sequence set forth in SEQ ID NO:1.

22. The polypeptide of claim 21, further comprising heterologous amino acid

24. A composition comprising the polypeptide of any one of claims 20, 21, and 22 and a pharmaceutically acceptable carrier.

25. A method for preventing, treating or ameliorating a medical condition which comprises administering to a mammalian subject a therapeutically effective amount of a composition of claim 24.

APPENDIX C

L34554
VERSION L34554.1 GI:509839
KEYWORDS .
SOURCE chicken.
ORGANISM Gallus gallus
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Archosauria; Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.
REFERENCE 1 (bases 1 to 1046)
AUTHORS Petrenko,O., Ischenko,I. and Enrietto,P.J.
TITLE Characterization of changes in gene expression associated with malignant transformation by the NF-kappaB family member, v-Rel
JOURNAL Oncogene 15 (14), 1671-1680 (1997)
MEDLINE 98007656
PUBMED 9349500
REFERENCE 2 (bases 1 to 1046)
AUTHORS Petrenko,O. and Enrietto,P.J.
TITLE Direct Submission
JOURNAL Submitted (15-JUL-1994) Molecular Biology, Princeton University, Princeton, NJ 08544, USA
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Revised: October 24, 2001.

LOCUS AAA49063 126 aa VRT 17-FEB-2000
DEFINITION stem cell antigen 2 [Gallus gallus].
ACCESSION AAA49063
PID g509840
VERSION AAA49063.1 GI:509840
DBSOURCE locus CHKSCA2A accession L34554.1
KEYWORDS .
SOURCE chicken.
ORGANISM Gallus gallus
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Archosauria; Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.
REFERENCE 1 (residues 1 to 126)
AUTHORS Petrenko,O., Ischenko,I. and Enrietto,P.J.
TITLE Characterization of changes in gene expression associated with malignant transformation by the NF-kappaB family member, v-Rel
JOURNAL Oncogene 15 (14), 1671-1680 (1997)
MEDLINE 98007656
PUBMED 9349500
REFERENCE 2 (residues 1 to 126)
AUTHORS Petrenko,O. and Enrietto,P.J.
TITLE Direct Submission
JOURNAL Submitted (15-JUL-1994) Molecular Biology, Princeton University, Princeton, NJ 08544, USA
COMMENT Method: conceptual translation.
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Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5296–5300, June 1994
Immunology

APPENDIX D

Mouse stem cell antigen Sca-2 is a member of the Ly-6 family of cell surface proteins

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Communicated by G. J. V. Nossal, February 15, 1994 (received for review November 19, 1993)

ABSTRACT **Mature T lymphocytes arise from intrathymic T-cell precursors, which in turn are derived from a multipotent stem cell in the bone marrow. Unlike bone marrow stem cells, the differentiation potential of the earliest intrathymic precursor cells is strongly biased toward the lymphoid lineage. The major difference in cell surface phenotype between early thymic precursor cells and bone marrow stem cells is that the former population expresses Sca-2. The progeny of the intrathymic precursor population continue to express Sca-2 until the transition from blast cells to small cells, at which stage expression of Sca-2 is down regulated. Mature thymocytes and peripheral T cells do not express detectable levels of Sca-2, whereas peripheral B cells are Sca-2-positive. We report herein the complete sequence of mouse Sca-2 deduced from a thymocyte cDNA clone. Sca-2 is a member of the Ly-6 family, a group of small cysteine-rich cell surface proteins that are anchored in the membrane by a glycosyl-phosphatidylinositol moiety.**

T lymphocytes develop from a multipotent stem cell that seeds the thymus from fetal liver and adult bone marrow (1, 2). A bone marrow stem cell population with the potential to reconstitute multiple hemopoietic lineages is also able to colonize an irradiated thymus with high efficiency after intrathymic transfer (3, 4). The earliest intrathymic precursor cells from adult thymus are found within a small population making up 0.05% of total thymocytes (5). Intrathymic transfer of this purified precursor population into irradiated recipients gives rise to T lymphocytes of the $\alpha\beta$ and $\gamma\delta$ lineages, with repopulation kinetics similar to that of thymus-seeding bone marrow progenitors (5). Since the intrathymic precursors have lost the ability to differentiate along the erythroid and myeloid lineage (6), they are the earliest known intrathymic lymphoid committed precursor. The cell surface phenotype of the intrathymic precursors is similar to that of bone marrow stem cells, except that the former express Sca-2 (6). Sca-2 is also expressed by the majority of immature thymocytes, but not by mature peripheral T cells. Sca-2 is also expressed on $B220^+$ bone marrow cells and peripheral B cells, including germinal center cells (7).

We report the complete primary structure of the Sca-2 molecule deduced from a cDNA clone isolated by expression cloning.† The Sca-2 molecule is a small Cys-rich protein anchored in the cell membrane via a glycosyl-phosphatidylinositol (GPI) moiety. Sca-2 is a member of the Ly-6 family, a group of molecules differentially expressed in several hematopoietic lineages that appears to function in signal transduction and cell activation.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs). The Sca-2 mAb E3 81-2.4 (8) was obtained from G. Spangrude (Laboratory of Persistent Viral Diseases, Hamilton, MT). Other mAbs used in this study were anti-Thy-1.2, clone 30H12 (9); anti-CD4, clone GK-1.5 (10); anti-CD3, clone KT3-1.1 (11); anti-intercellular adhesion molecule 1 (ICAM-1), clone 1.H4 (12); and anti-CD8, clone D9 (13).

Immunofluorescent Staining and Flow Cytometry Analysis. Two-stage labeling of cell surface molecules was carried out using appropriate mAb supernatant at a saturating dilution, followed after washing by incubation with affinity-purified F(ab')2 goat anti-rat IgG conjugated with fluorescein isothiocyanate (FITC) (Caltag, South San Francisco, CA) (7 μg/ml) or FITC-conjugated sheep anti-mouse IgG (Silenus, Hawthorn, Victoria, Australia) (7 μg/ml). Propidium iodide (1 μg/ml) was included in the final wash. Cell surface fluorescence was assessed by FACScan analysis (Becton Dickinson). Forward light scatter and propidium iodide staining gates were set to exclude dead cells.

Expression Cloning and Characterization. Sca-2 cDNA clones were isolated from a thymus cDNA library by using a COS-cell expression system (14, 15) and immunoselection with anti-Sca-2 mAb and sheep anti-rat immunoglobulin Dynabeads (Dynal, Oslo). Two rounds of DEAE/dextran transfection and selection were performed to isolate positive clones. Restriction enzyme fragments were subcloned into M13mp10 and M13mp18. Nucleotide sequencing was by the dideoxynucleotide chain-termination method and each nucleotide was determined in both directions with Sequenase dGTP and dITP reagents (United States Biochemical).

Protein Sequence Comparisons. Sequence comparisons were made with protein sequences obtained from the Protein Sequence Database of the Protein Identification Resource (Release 37.0, June 30, 1993), National Biomedical Research Foundation (Washington, DC) under the following accession numbers: RWHU59, human CD59; RWBEM3, herpesvirus CD59 homolog; A25708, mouse Ly-6E.1; A46528, ThB. Other sequences were translated from the following nucleotide sequences from National Center for Biotechnology Information–GenBank; MUSLY6C2A, mouse Ly-6C; MMLY6F112, mouse Ly-6F; MMLY6G113, mouse Ly-6G; RATLY6B; rat Ly-6B; RATLY6CA, rat Ly-6C. The squid glycoprotein (Sgp) 2 sequence was from ref. 16. The ALIGN program was used to determine statistical significance of sequence similarities (17), using the mutation data matrix with a matrix bias of +6, a gap penalty of 6, and 100 random runs.

Cleavage of GPI-Linked Cell Surface Molecules. Unfractionated thymocytes (2.5×10^6 cells) from female C57BL/6 mice were incubated with 190×10^{-3} units of phosphatidylinositol-specific phospholipase C (PI-PLC, Boehringer Mannheim) in 50 μl of Hepes-buffered mouse tonicity balanced salt solution (150 mM NaCl/3.7 mM KCl/2.5

Abbreviations: Sca, stem cell antigen; mAb, monoclonal antibody; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; ICAM-1, intercellular adhesion molecule

mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM K_2HPO_4/0.7 mM KH_2PO_4/15 mM Hepes, pH 7.2) at 37°C for 90 min. Cells were washed and then labeled with individual mAbs, followed by F(ab')2 goat anti-rat IgG conjugated with FITC. COS cells that had been transfected 2 days earlier with either Sca-2.1 plasmid or ICAM-1/CDM8 plasmid were detached from culture flasks with EDTA without trypsin and treated with PI-PLC as above, except that 6×10^5 cells were used per digest.

RESULTS

Isolation of Sca-2 cDNA Clones. Sca-2 cDNA clones were isolated using the COS-cell expression cloning protocol developed by Aruffo and Seed (14). A C57BL/6 thymocyte library in the πH3M vector (kindly provided by B. Seed, Massachusetts General Hospital, Boston) was introduced into COS-M6 cells by DEAE-dextran transfection. After 2 days in culture, cells were detached with EDTA, washed, and selected with the anti-Sca-2 mAb (E3 81-2.4) and paramagnetic Dynabeads coated with sheep anti-rat immunoglobulin (15). Episomal plasmid DNA was recovered from the adherent COS cells and used to transform *Escherichia coli* by electroporation. Plasmid DNA was purified from the resulting colonies and a second round of selection was performed, after which 98 colonies were individually picked and grown overnight in 1.5-ml cultures. After pooling the cultures in groups of seven, plasmid DNA was purified and transfected into COS cells. After two rounds of transfection and immunoselection, five out of eight pools were positive for Sca-2 expression by flow cytometry (data not shown). The seven individual plasmids making up one positive pool were transfected separately into COS cells and two out of seven gave Sca-2 expression (Fig. 1). The level of Sca-2 expression was heterogeneous with only a proportion of the cells clearly positive. However, this resembled the level of ICAM-1 expression after transfection with the control plasmid ICAM-1/CDM8, and both were clearly positive compared to the mock transfection (Fig. 1). A low level of cell surface expression was also seen for a murine HSA (CD24) clone isolated from the same library (unpublished data). Thus, despite the relatively low levels of expression, this protocol is suitable for the isolation of cDNA clones encoding cell surface antigens.

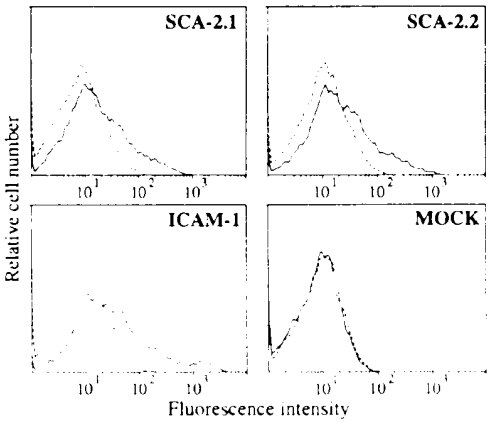


FIG. 1. Transient expression of Sca-2 on COS cells. Cells were transfected with the Sca-2.1 plasmid clone (*Upper Left*), the Sca-2.2 clone (*Upper Right*), or ICAM-1/CDM8 (*Lower Left*) or mock transfected (*Lower Right*) by the DEAE-dextran method. After 2 days, cells were detached and labeled with the anti-Sca-2 mAb (for Sca-2.1, Sca-2.2, and mock transfections, solid line) or with 1.H4 (anti-ICAM, solid line) for the ICAM transfection. The second-stage reagents were FITC-conjugated goat anti-rat IgG F(ab')2 for the

[illegible] ICAM transfection. Data from 10⁴ cells are shown for each histogram.

cDNA Sequence Analysis. Both positive plasmid clones contained cDNA inserts of ≈1.1 kb and had identical restriction maps (data not shown). One clone (Sca-2.1) was sequenced and the complete nucleotide sequence of 1072 bp is shown in Fig. 2*A*. The cDNA sequence contains two potential translational start sites (ATG) in the same reading frame at positions 44 and 62. The sequence after the first ATG codon shows an open reading frame coding for 136 amino acids (Fig. 2*B*). The 3′ untranslated region contains a consensus polyadenylylation site (AATAAA) 14 nucleotides from a poly(A) tail of 14 nucleotides [poly(A) tail not shown in Fig. 2*A*]. The translated sequence immediately downstream from initiator methionine codon at position 44 shows a high proportion of hydrophobic amino acid residues, characteristic of a posttranslationally cleaved leader sequence. The most likely cleavage point for this sequence is after the Ser residue at position −1 (Fig. 2*B*). There is a stretch of hydrophobic amino acid residues at the C terminus of the protein that is a signal sequence for attachment of a GPI anchor (see below).

The mature Sca-2 protein contains 82 amino acids with a predicted molecular mass of 8.8 kDa. This sequence contains a relatively high proportion of Cys residues (10/82), which suggests that numerous disulfide bonds stabilize the tertiary structure of Sca-2. There is one consensus sequence for N-linked oligosaccharide attachment (Asn-79). Ser-51 and Ser-56 are both flanked by Pro residues and may be O-glycosylated (19, 20).

Sequence Homology Between Sca-2 and the Ly-6 Family. A computer-assisted homology search of the protein database with the Sca-2 sequence revealed significant homology to members of the Ly-6 family (Fig. 3*A*). In the mouse, the Ly-6 family is encoded within a cluster of at least 18 genes on chromosome 15 (21), which includes ThB (22). Sca-2 shows significant structural similarities to mouse Ly-6C, Ly-6E.1, Ly-6F, Ly-6G, and ThB; rat Ly-6B and Ly-6C; human CD59; and a herpesvirus CD59 homolog. The level of amino acid identity between Sca-2 and other members of the Ly-6 family is relatively low. However, a remarkable conservation of all extracellular cysteine residues is evident, with all 10 Cys residues in the putative mature protein conserved between Sca-2 and each sequence shown in Fig. 3*A* (except Sgp-2, where 8 of 10 Cys residues are conserved). The statistical significance of the Sca-2 sequence alignments with members of the Ly-6 family was assessed using the ALIGN program. The optimal alignment between two sequences is computed and expressed as the number of SD units this alignment score is displaced from the mean optimal scores for the same sequences randomized. An alignment score of ≥3 SD is usually regarded as statistically significant, particularly if consistent scores are obtained with a number of sequences from the same family (17). The best alignment score for Sca-2 and a member of the Ly-6 family was with Ly-6G (8.5 SD), a recently defined molecule expressed in bone marrow (23). Other alignment scores shown in Fig. 3*B* clearly support an evolutionary relationship between Sca-2 and the Ly-6 family. The lowest alignment score between Sca-2 and any member of the Ly-6 family was for Sgp-2 (4.0 SD). Sgp-2 has been

Sca-2 Is Attached to the Cell Membrane by a GPI Anchor. The C terminus of the Sca-2 protein is enriched for hydro-

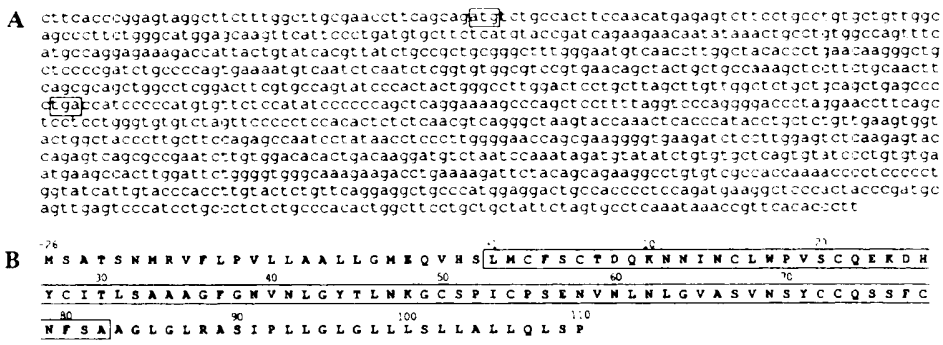
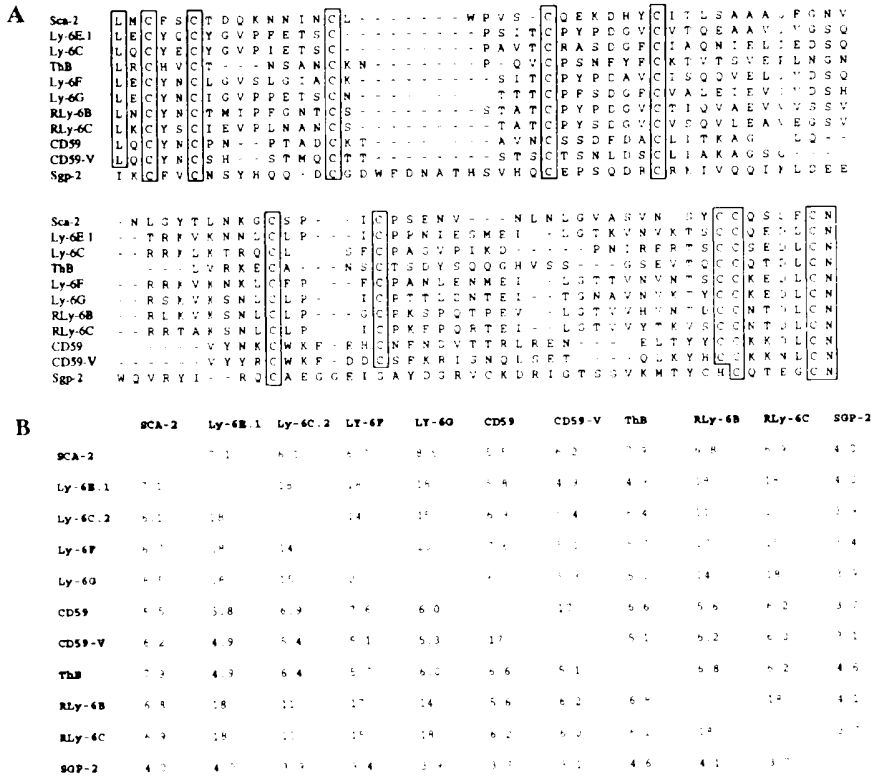


FIG. 2. (*A*) Nucleotide sequence of the thymocyte cDNA clone encoding mouse Sca-2. The methionine initiation codon and the stop codon are boxed. (*B*) Deduced amino acid sequence of Sca-2. The predicted sequence of the mature cell surface protein is boxed. The N-terminal amino acid, Leu-1, was assigned on the basis of homology to the Ly-6 sequences, and the C-terminal amino acid, Ala-82, was assigned according to GPI-attachment consensus sequences (18).

phobic amino acids, indicating that it is probably a signal peptide for the attachment of a GPI moiety for cell membrane anchoring (18). The overall sequence homology to the Ly-6 family supports this possibility, since all sequences shown in Fig. 3 have C-terminal hydrophobic signal sequences for GPI attachment or have a GPI anchor. The ability of PI-PLC to specifically cleave Sca-2 molecules from the surface of thymocytes and transfected COS cells was investigated. When thymocytes were treated with PI-PLC, a decrease in the mean cell surface expression of Sca-2 was seen, compared to the control sample in which cells were incubated for the same period without enzyme (Fig. 4*A*). In parallel samples, levels of a known GPI-anchored molecule (Thy-1) were also diminished, whereas the level of a non-GPI-anchored molecule (CD4) was unaffected (Fig. 4*A*). Sca-2 and Thy-1 vary in their susceptibility to cleavage by PI-PLC, which may reflect inherent structural differences between the two proteins or possibly steric constraints imposed by interactions with other unknown membrane molecules. The results of Fig. 4*B* show the specific cleavage of Sca-2 from the surface of Sca-2.1-transfected COS cells, in contrast to a parallel ICAM-1-transfected sample (a non-GPI-anchored molecule), which



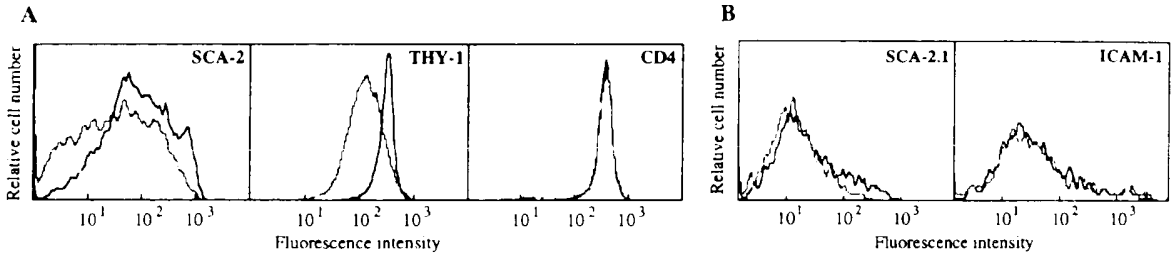


FIG. 4. Sca-2 is attached to the cell membrane by a GPI anchor. (*A*) Thymocytes were incubated at 37°C in the presence (vertical hatched lines) or absence (solid lines) of PI-PLC. Cells were labeled with the mAbs indicated then with FITC-conjugated anti-IgG as described in Fig. 1. The negative staining profile obtained with an isotype-matched negative control mAb is shown (stippled line). Data from 10^4 cells are shown for each histogram. (*B*) Sca-2.1-transfected COS cells (*Left*) or ICAM/CDM8-transfected COS cells (*Right*) were incubated in the presence or absence of PI-PLC and stained as above. Data from 3×10^3 cells are shown for each histogram.

was unaffected by PI-PLC. These results confirm that the predicted GPI signal sequence (Fig. 2*B*) directs attachment of a GPI anchor to the C terminus of Sca-2 and that this modification occurs in both thymocytes and COS cells. The conservation of the Cys-Asn sequence near the C terminus of the mature protein in all members of the Ly-6 family (Fig. 3*A*) suggests a consensus attachment point for the GPI anchor, since the corresponding Asn residue in Sgp-2 has been empirically determined as the C-terminal amino acid (16). However, data for known GPI attachment sites suggests that Ala-82 is the C-terminal amino acid of Sca-2 (18).

DISCUSSION

The Sca-2 antigen is a small Cys-rich cell surface protein of 82 amino acids and is a member of the Ly-6 family. Sca-2 is anchored in the cell membrane by a C-terminal GPI moiety, a posttranslational modification in common with each member of the Ly-6 family described to date. Several GPI-linked molecules have been implicated in signal transduction in hematopoietic cells, suggesting an important role for the GPI anchor in cell activation (24). For Ly-6A/E, mAb ligation induced a mitogenic signal to T cells in the presence of accessory cells or interleukin 1 (25, 26). Signal transduction in T cells via GPI-anchored proteins involves interleukin 2 production and this is dependent on the presence of a full-length T-cell receptor ζ chain (27). However, one report has shown that Ly-6A/E mAbs can induce a down regulation of an interleukin 2 response (28). It is possible that Sca-2 may also function as a signal transduction molecule, and the presence of a GPI anchor suggests a signaling mechanism in common with other GPI-anchored molecules.

Sca-2 is also related to human CD59, an Ly-6-related protein of broad tissue distribution that inhibits complement-mediated cytolysis of erythrocytes and leukocytes by competing with C9 incorporation into the membrane attack complex (30, 31). CD59 has been proposed as a second cell surface ligand for the CD2 antigen (32), although a recent report (33) shows that a multivalent form of soluble CD2 does not bind CD59. Another member of the Ly-6 family (not shown in Fig. 3) is the urokinase-type plasminogen activator receptor (uPAR), a major cell surface regulator of plasminogen activation (34). The uPAR sequence reveals three Cys-rich Ly-6-like domains compared to the single domain found in all other Ly-6 family members (35–37). The ligand binding activity of uPAR is contained within the N-terminal Ly-6-like domain, which binds an epidermal growth factor-like domain in urokinase-type plasminogen activator (35, 38). A sequence comparison of Sca-2 with the N-terminal [illegible] ligands with epidermal growth factor like domains [illegible]

recent report has shown structural homology between the Ly-6 family and several snake toxins (23), which suggests an important structural role for this domain in proteins of diverse function.

The Sca-2 antigen is expressed on immature thymocytes but not on mature thymocytes or peripheral T cells. Sca-2 is not confined to the T lineage, since B-lineage cells in the bone marrow and the periphery are also Sca-2$^+$. Nevertheless, within the T lineage, upregulation of Sca-2 expression appears to coincide with the transition from a multipotential bone marrow stem cell to an intrathymic lymphoid committed precursor cell (6). Further characterization of this lymphoid precursor is central to the understanding of early T-cell differentiation and lymphoid lineage commitment. The expression of Sca-2 by a lymphoid precursor cell upon entry into the thymus suggests an important function for Sca-2 in early thymopoiesis.

Note. While this manuscript was under review, the sequence of a mouse thymocyte antigen, TSA-1, was reported (29). The deduced protein sequence of TSA-1 is identical to the Sca-2 sequence reported herein, except that we report a Gly residue in the N-terminal leader sequence at position −7 whereas MacNeil *et al.* (29) report an Arg at this position.

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APPENDIX E

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Medical Sciences

Prostate stem cell antigen: A cell surface marker overexpressed in prostate cancer

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Contributed by Owen N. Witte, December 24, 1997

ABSTRACT **The identification of cell surface antigens is critical to the development of new diagnostic and therapeutic modalities for the management of prostate cancer. Prostate stem cell antigen (PSCA) is a prostate-specific gene with 30% homology to stem cell antigen 2, a member of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored cell surface antigens. PSCA encodes a 123-aa protein with an amino-terminal signal sequence, a carboxyl-terminal GPI-anchoring sequence, and multiple N-glycosylation sites. PSCA mRNA expression is prostate-specific in normal male tissues and is highly up-regulated in both androgen-dependent and -independent prostate cancer xenografts. *In situ* mRNA analysis localizes PSCA expression in normal prostate to the basal cell epithelium, the putative stem cell compartment of the prostate. There is moderate to strong PSCA expression in 111 of 126 (88%) prostate cancer specimens examined by *in situ* analysis, including high-grade prostatic intraepithelial neoplasia and androgen-dependent and androgen-independent tumors. Flow cytometric analysis demonstrates that PSCA is expressed predominantly on the cell surface and is anchored by a GPI linkage. Fluorescent *in situ* hybridization analysis localizes the PSCA gene to chromosome 8q24.2, a region of allelic gain in more than 80% of prostate cancers. A mouse homologue with 70% amino acid identity and similar genomic organization to human PSCA has also been identified. These results support PSCA as a target for prostate cancer diagnosis and therapy.**

Prostate cancer is the most common cancer diagnosis and the second leading cause of cancer-related death in American men. Despite recent advances in the detection and treatment of localized disease, significant challenges remain in the management of this disease. Current diagnostic modalities are limited by a lack of specificity and an inability to predict which patients are at risk to develop metastatic disease. Prostate-specific antigen (PSA) is effective at identifying men who may have prostate cancer but is often elevated in men with benign prostatic hyperplasia, prostatitis, and other nonmalignant disorders (1). PSA and other current markers fail to discriminate accurately between indolent and aggressive cancers. There is no effective treatment for the 20–40% of patients who develop recurrent disease after surgery or radiation therapy or for those who have metastatic disease at the time of diagnosis. Although hormone ablation therapy can palliate these pa-

In an effort to identify potential markers for the diagnosis and treatment of prostate cancer, we have searched for genes up-regulated during prostate cancer progression by using the recently developed LAPC-4 xenograft model of human prostate cancer (2). The LAPC-4 system accurately recapitulates many of the features of advanced human prostate cancer, including progression to androgen independence and metastasis. We have sought to identify genes encoding secreted or cell surface proteins, because they have potential utility as serum markers of prostate cancer, similar to PSA and glandular kallikrein 2, and may be useful for detecting or targeting prostate cancer cells (3). Prostate specific membrane antigen (PSMA) is a recently described cell surface marker of prostate cancer currently being evaluated for the detection of metastatic cells and as a target for mAb and other immunological therapies (4, 5). Another potential advantage to searching for membrane-bound tumor antigens is that they may provide insights into the biology of prostate cancer progression. Her-2/neu, one of a number of growth factor receptors associated with prostate cancer, is being evaluated both as a target for therapy and as an important signaling molecule in androgen-independent prostate cancer (6–8). Other receptors, such as c-met and urokinase plasminogen activator receptor, may mediate prostate cancer metastasis (9–11).

We have identified a number of candidate molecules up-regulated in the LAPC-4 xenograft model by using representational difference analysis (RDA), a PCR-based subtractive hybridization strategy (12). One promising candidate, prostate stem cell antigen (PSCA), is a prostate-specific cell surface antigen expressed strongly by both androgen-dependent and -independent LAPC-4 tumors. PSCA is homologous to a group of cell surface proteins that mark the earliest phases of hematopoietic development. We hypothesize that PSCA may play a role in prostate cancer progression and may serve as a target for prostate cancer diagnosis and treatment.

MATERIALS AND METHODS

Molecular Studies. RDA of androgen-dependent and -independent LAPC-4 tumors was performed as described (13). Total RNA was isolated by using Ultraspec RNA isolation systems (Biotecx, Houston, TX) according to the manufacturer's instructions. Northern blot filters were probed with a

Abbreviations: PSA, prostate-specific antigen; PSMA, prostate-

[illegible]

[illegible]

660-bp RDA fragment corresponding to the coding sequence and part of the 3′ untranslated sequence of PSCA or an ~400-bp fragment of PSA. The human multiple tissue blot was obtained from CLONTECH and probed as specified. For reverse transcriptase-coupled PCR (RT-PCR) analysis, first-strand cDNA was synthesized from total RNA by using the GeneAmp RNA PCR core kit (Perkin–Elmer–Roche). For RT-PCR of human PSCA transcripts, primers 5′-TGCTTGCCCTGTTGATGGCAG and 3′-CCAGAGCAGCAGGCCGAGTGCA were used to amplify an ~320-bp fragment. Thermal cycling was performed for 25 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, followed by extension at 72°C for 10 min. Primers for GAPDH (CLONTECH) were used as controls. For mouse PSCA, the primers used were 5′-TTCTCCTGCTGGCCACCTAC and 3′-GCAGCTCATCCCTTCACAAT.

For mRNA *in situ* hybridization, recombinant plasmid pCR II (1 μg, Invitrogen) containing the full-length PSCA gene was linearized to generate sense and antisense digoxigenin-labeled RNA probes. *In situ* hybridization was performed on an automated instrument (Ventana Gen II, Ventana Medical Systems) as described (14). Prostate specimens were obtained from a previously described database that has been expanded to ~130 specimens (14). Slides were read and scored by two pathologists in a blinded fashion. Scores of 0–3 were assigned according to the percentage of positive cells (0 = 0%; 1 = <25%; 2 = 25–50%; 3 = >50%) and the intensity of staining (0 = 0; 1 = 1+; 2 = 2+; 3 = 3+). The two scores were multiplied to give an overall score of 0–9.

λ phage clones containing the human PSCA gene were obtained by screening a human genomic library (Stratagene) with a human PSCA cDNA probe (15). BAC (bacterial artificial chromosome) clones containing the murine PSCA gene were obtained by screening a murine BAC library (Genome Systems, St. Louis). A 14-kb human *Not*I fragment and a 10-kb murine *Eco*R1 fragments were subcloned into pBluescript (Stratagene) and subjected to restriction mapping. Fluorescence *in situ* chromosomal analysis was performed as described by using overlapping human λ phage clones (16).

Biochemical Studies. Rabbit polyclonal antiserum was generated against the synthetic peptide TARIRAVGLLTVISK and affinity-purified by using a PSCA–glutathione *S*-transferase fusion protein. 293T cells were transiently transfected with pCDNA II (Invitrogen) expression vectors containing PSCA, CD59, E25, or vector alone by calcium phosphate precipitation. Immunoprecipitation was performed as described (17). Briefly, cells were labeled with 500 μCi of trans-^{35}S label (ICN) for 6 h. Cell lysates and conditioned medium were incubated with 1 μg of purified rabbit anti-PSCA antibody and 20 μl of protein A-Sepharose CL-4B (Pharmacia Biotech) for 2 h. For deglycosylation, immunoprecipitates were treated overnight at 37°C with 1 unit of *N*-glycosidase F (Boehringer Mannheim) or 0.1 unit of neuraminidase (Sigma) for 1 h followed by an overnight incubation in 2.5 milliunits of *O*-glycosidase (Boehringer Mannheim).

For flow cytometric analysis of PSCA cell surface expression, single cell suspensions were stained with purified anti-PSCA antibody (2 μg/ml) and a 1:500 dilution of phycoerythrin-labeled anti-rabbit IgG (Jackson ImmunoResearch). DNA was acquired on a FACScan (Becton Dickinson) and

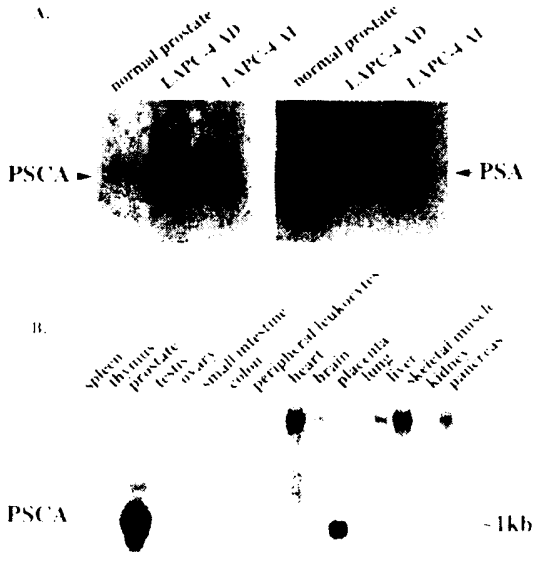
[illegible] (PI-PLC, Boehringer Mannheim) for 90 min at 37°C. Cells were analyzed prior to and after digestion by either flow cytometry scanning or immunoblotting.

RESULTS

RDA Identifies PSCA. RDA was used to isolate cDNAs up-regulated in an androgen-independent subline of the LAPC-4 prostate cancer xenograft (12). In the course of this work, one 660-bp fragment (clone 15) was identified that was found to be highly overexpressed in xenograft tumors when compared with normal prostate but was not differentially expressed between androgen-dependent and -independent LAPC-4 tumors (Fig. 1*A*). Comparison of the expression of this clone to that of PSA in normal prostate and xenograft tumors suggested that clone 15 was relatively cancer-specific (Fig. 1*A*).

Sequence analysis revealed that clone 15 had no exact match in the databases but shared 30% nucleotide homology with stem cell antigen 2 (SCA-2), a member of the Thy-1/Ly-6 superfamily of GPI-anchored cell surface antigens. Clone 15 encodes a 123-amino acid protein that is 30% identical to SCA-2 (also called RIG-E) and contains a number of highly conserved cysteine residues characteristic of the Ly-6/Thy-1 gene family (Fig. 2) (18). Consistent with its homology to a family of GPI-anchored proteins, clone 15 contains both an amino-terminal hydrophobic signal sequence and a carboxyl-terminal stretch of hydrophobic amino acids preceded by a group of small amino acids defining a cleavage/binding site for GPI linkage (19). It also contains four predicted N-glycosylation sites. Because of its strong homology to SCA-2, clone 15 was renamed PSCA.

The human PSCA cDNA was used to search murine expressed sequence tag (EST) databases to identify homologues for potential transgenic and knockout experiments. One EST obtained from fetal mouse and another from neonatal kidney were 70% identical to the human cDNA at both the nucleotide and amino acid levels. The homology between the mouse clones and human PSCA included regions of divergence between human PSCA and its GPI-anchored homologues, indicating that these clones likely represented the mouse homologue of PSCA. Alignment of these ESTs and 5′ exten-



[illegible] 28S RNA as the loading control. *B*, Human multiple tissue Northern blot analysis of PSCA. The filter was obtained from CLONTECH and contains 2 μg of poly(A)$^+$ RNA in each lane.

```
1   MKIFLPVLLAALLGVERASS  hSCA-2
1   MKAVLLALLMAGLALQPGTA  hPSCA
1   MKTVLFLLLATYLALHPGAA  mPSCA

21  LMCFSCLNQKSN*LYCLKPTI
21  LLCYSCKAQVSN*EDCLQVEN*
21  LQCYSCTAQMNN*RDCLNVQN*

41  CSDQDNYCVTVSASAGIGNL
41  CTQLGEQCWTARIRAVGLLT
41  CSLDQHSCFTSRIRAIGLVT

61  VTFGHSLSKTCSPACPIPEG
61  V-----ISKGCSLNCVDDSQ
61  V-----ISKGCSSQCEDDSE

81  VNVGVASMGISCCQSFLCN*F
76  DYYVGKK-N*ITCCDTDLCN*A
76  NYYLGKK-N*ITCCYSDLCN*V

101 SAADGGLRASVTLLGAGLLL
95  SGAHALQPAAAILALLPALG
95  NGAHTLKPPTTLGLLTVLCS

121 SLLPALLRFGP
115 LLLWGPGQL--
115 LLLWGSSRL--
```

FIG. 2. Amino acid sequences of human SCA-2 (hSCA-2), human PSCA (hPSCA), and mouse PSCA (mPSCA). Shaded regions highlight conserved amino acids, and conserved cysteines are indicated by boldface type. Four predicted N-glycosylation sites in PSCA are indicated by asterisks. The underlined amino acids at the beginning and end of the protein represent amino-terminal hydrophobic signal sequences and C-terminal GPI-anchoring sequences, respectively.

sion by using rapid amplification of cDNA ends coupled to PCR provided the entire coding sequence (see Fig. 2).

PSCA Expression Is Prostate-Specific. The distribution of PSCA mRNA in normal human tissues was examined by Northern blot analysis. PSCA is expressed predominantly in prostate, with a lower level of expression present in placenta (Fig. 1*B*). Small amounts of mRNA can be detected in kidney and small intestine after prolonged exposure and are approximately 1% of the level seen in prostate. RT-PCR analysis of PSCA expression in normal human tissues produced similar results (data not shown). The major PSCA transcript in normal prostate is ~1 kb (Fig. 1*B*). Mouse PSCA expression was analyzed by RT-PCR in mouse spleen, liver, lung, prostate, kidney, and testis (data not shown). Like human PSCA, murine PSCA is expressed predominantly in prostate. Expression can also be detected in kidney at a level similar to that seen for placenta in human tissues. These data indicate that PSCA expression is largely prostate-specific.

PSCA Is Expressed by a Subset of Basal Cells in Normal Prostate. Normal prostate contains two major epithelial cell populations—secretory luminal cells and subjacent basal cells. *In situ* hybridizations were performed on multiple sections of normal prostate by using an antisense RNA probe specific for PSCA to localize its expression. PSCA is expressed exclusively in a subset of normal basal cells (Fig. 3*A*). Little to no staining is seen in stroma, secretory cells, or infiltrating lymphocytes. Hybridization with sense PSCA RNA probes showed no background staining. Hybridization with an antisense probe for GAPDH confirmed that the RNA in all cell types was intact. Because basal cells represent the putative progenitor cells for the terminally differentiated secretory cells, these

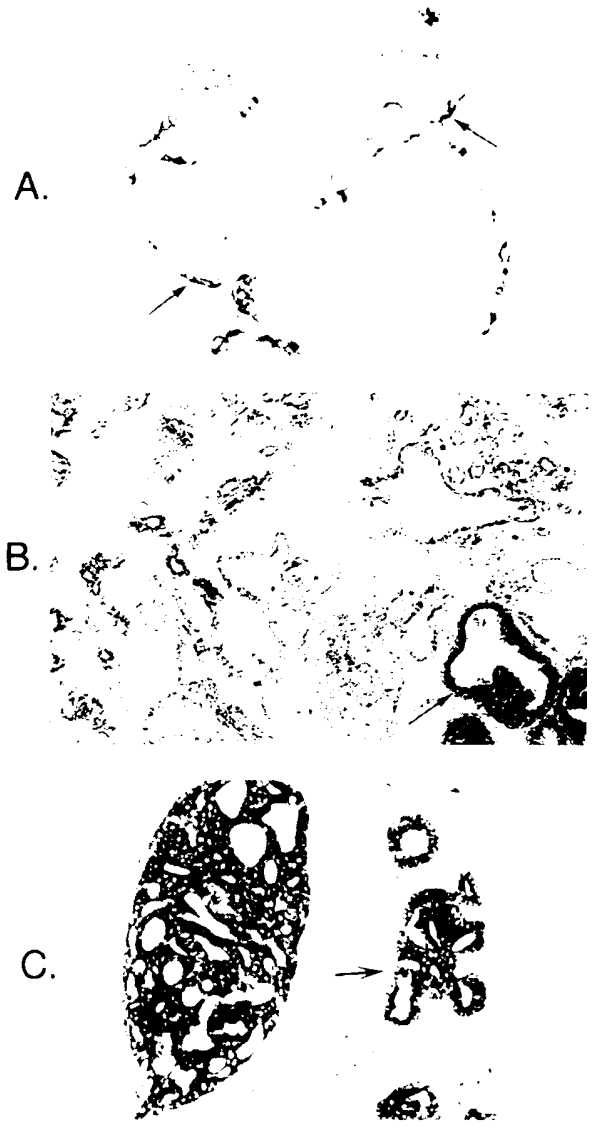


FIG. 3. *In situ* hybridization with antisense RNA probe for human PSCA on normal and malignant prostate specimens. (*A*) PSCA is expressed by a subset of basal cells within the basal cell epithelium (black arrows) but not by the terminally differentiated secretory cells lining the prostate ducts. (×400.) (*B*) PSCA is expressed strongly by a high-grade prostatic intraepithelial neoplasia (black arrow) and by invasive prostate cancer glands (yellow arrows) but is not detectable in normal epithelium (green arrow) at ×40 magnification. (*C*) Strong expression of PSCA in high-grade carcinoma. (×200.)

tumors removed by radical prostatectomy or transurethral resection in all cases except one. All specimens were probed with both a sense and antisense construct to control for background staining. Slides were assigned a composite score, with a score of 6 to 9 indicating strong expression and a score of 4 meaning moderate expression. One hundred two of 126

suggested that PSCA was not expressed in prostatic atrophy. One hundred twenty-six paraffin-embedded prostate cancer specimens were analyzed by mRNA *in situ* hybridization for PSCA expression. Specimens were obtained from primary

tant glands (Fig. 3*B*). Nine specimens were obtained from patients treated before surgery with hormone ablation therapy. Seven of nine (78%) of these residual presumably andro-

gen-independent cancers overexpressed PSCA, a percentage similar to that seen in untreated cancers. Because such a large percentage of specimens expressed PSCA mRNA, no statistical correlations could be made between PSCA expression and pathological features such as tumor stage and grade. These results suggest that PSCA mRNA overexpression is a common feature of androgen-dependent and -independent prostate cancer.

PSCA expression was also detected in the androgen-independent androgen-receptor-negative prostate cancer cell lines PC3 and DU145 by RT–PCR analysis (data not shown). These data suggest that PSCA can be expressed in the absence of functional androgen receptor.

PSCA Is a GPI-Anchored Glycoprotein Expressed on the Cell Surface. The deduced PSCA amino acid sequence predicts that PSCA is heavily glycosylated and anchored to the cell surface through a GPI mechanism. To test these predictions, we produced an affinity-purified polyclonal antibody raised against a unique PSCA peptide. This peptide contains no glycosylation sites and was predicted, based on a comparison to the three-dimensional structure of CD59 (another GPI-anchored PSCA homologue), to lie in an exposed portion of the mature protein (22). Recognition of PSCA by the affinity-purified antibody was demonstrated by immunoblot and immunoprecipitation analysis of a glutathione *S*-transferase–PSCA fusion protein and extracts of 293T cells transfected with PSCA. The polyclonal antibody immunoprecipitates predominantly a 24-kDa band from PSCA-transfected but not mock-transfected cells (Fig. 4*A*). Three smaller bands are also present, the smallest being ≈10 kDa. The immunoprecipitate was treated with N- and O-specific glycosidases to determine whether these bands represented glycosylated forms of PSCA. *N*-glycosidase F deglycosylated PSCA, whereas *O*-glycosidase had no effect (Fig. 4*A*). Some GPI-anchored proteins are known to have both membrane-bound and secreted forms (23, 24). Fig. 4*B* indicates that some PSCA is secreted in the 293T-overexpressing system. The secreted form of PSCA migrates at a lower molecular weight than the cell surface-associated form, perhaps reflecting the absence of the covalent GPI-linkage. This result may reflect the high level of expression in the 293T cell line and needs to be confirmed in prostate cancer cell lines and *in vivo*.

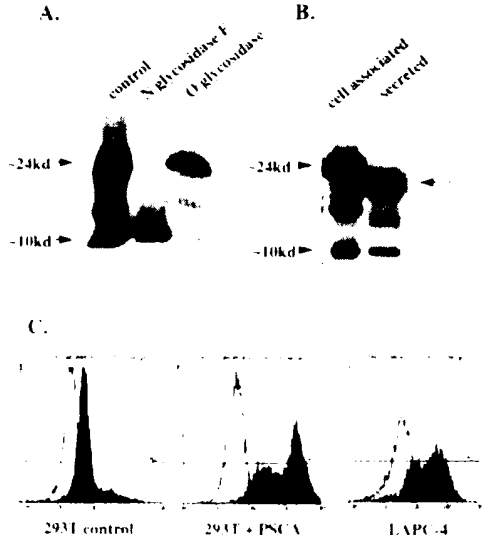


FIG. 4. Biochemical analysis of PSCA. (*A*) PSCA was immunoprecipitated from 293T cells transiently transfected with a PSCA [illegible]

[illegible] using affinity-purified polyclonal anti-PSCA antibody. Cells were not permeabilized to detect cell surface expression. The *x* axis represents relative cell number and the *y* axis represents fluorescent staining intensity on a logarithmic scale.

Fluorescence-activated cell sorting analysis was used to localize PSCA expression to the cell surface. Nonpermeabilized mock-transfected 293T cells, PSCA-expressing 293T cells, and LAPC-4 cells were stained with affinity-purified antibody or secondary antibody alone. Fig. 4*C* shows cell surface expression of PSCA in PSCA-transfected 293T and LAPC-4 cells but not in mock-transfected cells. To confirm that this cell surface expression is mediated by a covalent GPI linkage, cells were treated with GPI-specific PLC. Release of PSCA from the cell surface by PLC was indicated by more than a one order of magnitude reduction in fluorescence intensity (data not shown). Recovery of PSCA in postdigest conditioned medium was also confirmed by immunoblotting (data not shown). The specificity of PLC digestion for GPI-anchored proteins was confirmed by performing the same experiment on 293T cells transfected with the GPI-linked antigen CD59 or the non-GPI-linked transmembrane protein E25a (25). PLC digestion reduced cell surface expression of CD59 to the same degree as PSCA but had no effect on E25. These results support the prediction that PSCA is a glycosylated GPI-anchored cell surface protein.

The PSCA Gene Maps to Chromosome 8q24.2. Southern blot analysis of LAPC-4 genomic DNA revealed that PSCA is encoded by a single copy gene (data not shown). Other Ly-6 gene family members contain four exons, including a first exon encoding a 5′ untranslated region and three additional exons encoding the translated and 3′ untranslated regions. Genomic clones of human and murine PSCA containing all but the presumed 5′ first exon were obtained by screening λ phage libraries. Mouse and human PSCA clones had a similar genomic organization. The human clone was used to localize PSCA by fluorescence *in situ* hybridization analysis. Cohybridization of overlapping human PSCA λ phage clones resulted in specific labeling only of chromosome 8 (Fig. 5). Ninety-seven percent of detected signals localized to chromosome 8q24, of

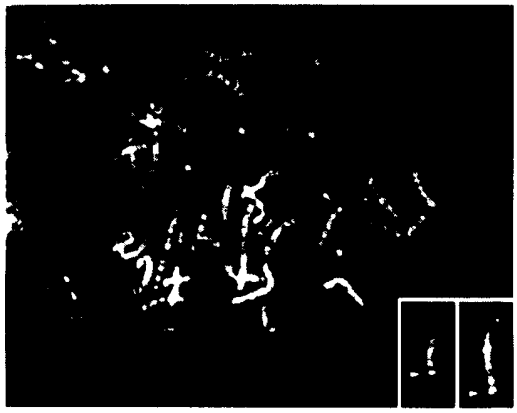


FIG. 5. *In situ* hybridization of biotin-labeled PSCA probes [illegible]

[illegible] microscope equipped [illegible] camera. Separate images of 4′,6-diamidino-2-phenylindole stained chromosomes and the hybridization signal were merged by using image analysis software (SV 200 and IMAGE 1.57).

which 87% were specific for chromosome 8q24.2. These results show that PSCA is located at chromosome 8, band q24.2.

DISCUSSION

PSCA is a prostate cancer-associated tumor antigen. Northern blot and *in situ* data show that PSCA is predominantly prostate-specific in normal tissues and is overexpressed in greater than 80% of prostate cancers. The expression of PSCA in cancers appears to be the reverse of what is seen with PSA. Although PSA is expressed more strongly in normal than malignant prostate, PSCA is expressed more highly in prostate cancer. This relationship is clearly seen in the case of the LAPC-4 xenograft tumors and is inferred from the large number of *in situ* hybridizations performed. These data suggest that PSCA may be a useful marker for discriminating cancers from normal glands in prostatectomy specimens.

The cell surface location of PSCA makes it a putative target for therapy and diagnosis. One possibility is that antibodies directed against PSCA may be used to locate metastatic disease in patients considering local therapy or in those believed to have metastatic disease, analogous to the Prostascint test currently being developed by using an antibody against PSMA (26). Antibodies against PSCA might be used to deliver radioisotopes or other toxins specifically to prostate cancer cells. Finally, PSCA could be tested as an immunogen for various immunological therapies. Similar approaches are currently under development for PSMA (4, 27).

A preliminary survey of 126 prostate cancer specimens did not reveal any clear correlation of PSCA expression with cancer grade, stage, or hormone dependency but rather showed widespread overexpression of this marker. PSCA expression was maintained in most androgen-independent tumors and was also detected in androgen-receptor negative cell lines, suggesting that PSCA may a useful marker of androgen-independent tumors that have lost functional androgen receptor. It should be emphasized that the primary goal of this survey was to demonstrate expression of PSCA mRNA *in vivo*. Also, metastatic prostate cancers were not studied other than the LAPC-4 xenograft, which was derived from a lymph node metastasis. Additional studies looking at PSCA protein expression may provide important clinical information not attainable by *in situ* hybridization. In a recent study on thymosin β15 expression, RNA *in situ* analyses were consistently positive, whereas immunohistochemical analysis revealed a possible correlation between Gleason grade and level of thymosin β15 expression (28).

An intriguing finding in this study was the localization of PSCA mRNA to a subset of basal cells in normal prostate. The basal cell epithelium is believed to contain the progenitor cells for the terminally differentiated secretory cells (20). Recent studies using cytokeratin markers suggest that the basal cell epithelium contains at least two distinct cellular subpopulations, one expressing cytokeratins 5 and 14 and the other cytokeratins 5, 8, and 18 (29). The finding that PSCA is expressed by only a subset of basal cells suggests that PSCA may be a marker for one of these two basal cell lineages.

A number of investigators have hypothesized that prostate cancers may arise from transformation of basal cells (30). Verhagen *et al.* (31) identified a population of cells in prostate cancers that coexpress basal and secretory cell cytokeratins 5, [illegible] present. Differences in RNA and protein localization have been noted previously for androgen receptor and PSMA expression (33, 34).

The biological function of PSCA is not known. The Ly-6 gene family is involved in diverse cellular functions, including signal transduction and cell–cell adhesion. Signaling through SCA-2 has been demonstrated to prevent apoptosis in immature thymocytes (35). Thy-1 is involved in T cell activation and transmits signals through src-like tyrosine kinases (36). Ly-6 genes have been implicated both in tumorigenesis and in homotypic cell adhesion (37–39). From its restricted expression in basal cells and its homology to SCA-2, we hypothesize that PSCA may play a role in stem/progenitor cell functions such as self-renewal (antiapoptosis) and/or proliferation.

PSCA is highly conserved in mice and humans. The identification of a conserved gene that is predominantly restricted to prostate supports the hypothesis that PSCA may play an important role in normal prostate development and should be evaluated by creation of transgenic and knockout models.

PSCA maps to chromosome 8q24.2. Human SCA-2 (also called RIG-E) and another recently identified human Ly-6 homologue (i.e., E48) also localize to this region, suggesting that a large family of related genes may exist at this locus (18, 39). Intriguingly, chromosome 8q has been reported to be a region of allelic gain and amplification in a majority of advanced and recurrent prostate cancers (40). c-*myc* localizes proximal to PSCA at chromosome 8q24 and extra copies of c-*myc* (either through allelic gain or amplification) have been found in 68% of primary prostate tumors and 96% of metastases (41). Additional work will be necessary to see if PSCA overexpression is caused by amplification or chromosomal gain.

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[illegible] work clearly will be needed to prove this hypothesis. It should be particularly important to study PSCA expression at the protein level, because it remains possible that although PSCA mRNA is not detected in secretory cells, PSCA protein may be

[illegible]

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APPENDIX F

lalign output for SEQ ID NO:1 USSN 09/529,205 vs. Translation of AF043498

Reiter et al PNAS 95:1735

[ISREC-Server] Date: Thu Dec 13 0:31:35 MET 2001

LALIGN finds the best local alignments between two sequences version 2.0u66 September 1998 Please cite: X. Huang and W. Miller (1991) Adv. Appl. Math. 12:373-381

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 Comparison of:
(A) ./wwwtmp/lalign/.8289.1.seq SEQ ID NO:1  USSN 09/529,205                    -
(B) ./wwwtmp/lalign/.8239.2.seq Translation of AF043498 Reiter et al PNAS 95:1735  -
 using matrix file: BL50, gap penalties: -14/-4

  99.2  identity in 123 aa overlap; score:  834 E(10,000): 1.9e-68

               10        20        30        40        50        60
SEQ     MKAVLLALLMAGLALQPGTALLCYSCKAQVSNEDCLQVKNCTQLGEQCWTARIRAVGLLT
        ::::::::::::::::::::::::::::::::::::::::.:::::::::::::::::::
Transl  MKAVLLALLMAGLALQPGTALLCYSCKAQVSNEDCLQVENCTQLGEQCWTARIRAVGLLT
               10        20        30        40        50        60

               70        80        90       100       110       120
SEQ     VISKGCSLNCVDDSQDYYVGKKNITCCDTDLCNASGAHALQPAAAILALLPALGLLLWGP
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Transl  VISKGCSLNCVDDSQDYYVGKKNITCCDTDLCNASGAHALQPAAAILALLPALGLLLWGP
               70        80        90       100       110       120


SEQ     SQL
        :::
Transl  SQL
```

```
 [illegible] identity in [illegible] aa overlap; score: [illegible] E(10,000): [illegible]


[illegible]     [illegible]
        ::::..:.:
Transl  AL[illegible]AAAIL
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```
 [illegible] identity in [illegible] aa overlap; score: [illegible] E(10,000): [illegible]
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